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# Roles of outer capsid proteins as determinants of pathogenicity and host range restriction of avian rotaviruses in a suckling mouse model

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**Abstract**

We previously demonstrated that a pigeon rotavirus, PO-13, but not turkey strains Ty-3 and Ty-1 and a chicken strain, Ch-1, induced diarrhea in heterologous suckling mice. In this study, it was suggested that these avirulent strains, but not PO-13, were inactivated immediately in gastrointestinal tracts of suckling mice when they were orally inoculated. To determine which viral proteins contribute to the differences between the pathogenicitiy and the inactivation of PO-13 and Ty-3 in suckling mice, six PO-13 × Ty-3 reassortant strains that had the genes of the outer capsid proteins, VP4 and VP7, derived from the opposite strain were prepared and were orally inoculated to suckling mice. A single strain that had both PO-13 VP4 and VP7 with the genetic background of Ty-3 had an intermediate virulence for suckling mice. Three strains with Ty-3 VP7, regardless of the origin of VP4, rapidly disappeared from gastrointestinal tracts of suckling mice. These results indicated that the difference between the pathogenicity of PO-13 and that of Ty-3 was mainly dependent on both their VP4 and VP7. In particular, VP7 was found to be related to the inactivation of Ty-3 in gastrointestinal tracts of suckling mice.


*Keywords:* Pathogenicty; Host range restriction; Avian rotaviruses; Inactivation; Reassortant strains; VP7

## Introduction

Group A rotaviruses (rotaviruses), members of the family Reoviridae, are recognized as the main cause of acute gastroenteritis in infants and young mammalian animals (Estes and Cohen, 1989). Although the majority of rotaviruses are known to be species-specific, several epidemiological surveys (Gerna et al., 1992; Nakagomi et al., 1990; Pongsuwanna et al., 1996; Urasawa et al., 1992) and experiments in which animals were infected with heterologous viruses (Bell et al., 1987; Ciarlet et al., 1998; Schwers et al., 1983) have demonstrated that some rotaviruses are transmitted to other species, including humans. Rotaviruses have also been isolated from several avian species (Legrottaglie et al., 1997; McNulty et al., 1980; Minamoto et al., 1988). It is known that rotaviruses induce severe diarrhea, sometimes resulting in death, in turkeys and pheasants (Legrottaglie et al., 1997; McNulty et al., 1980). Several studies have suggested that avian rotaviruses had divided from mammalian rotaviruses at an early stage of the evolutionary process (Ito et al., 1995, 1997, 2001). Thus, it has been doubtful that interspecies transmissions of avian rotaviruses to mammals could occur in nature. However, the avianlike strain 993/83 has been isolated from the feces of a calf suffering from diarrhea in Germany (Brüssow et al., 1992a). This virus was more similar to avian rotaviruses than to mammalian rotaviruses in terms of genetic and antigenic properties (Brüssow et al., 1992a,1992b; Ito et al., 1997; Rohwedder et al., 1995, 1997). A seroepidemiological survey showed that the virus has been prevalent in calves in Germany (Brüssow et al., 1992b). Furthermore, the pigeon virus strain PO-13 has been found to be infectious to and virulent in heterologous suckling mice (Mori et al., 2001). These findings suggest that avian rotaviruses play a role as cross-species pathogens in avian and mammalian species. On the other hand, we also found that turkey virus strains Ty-3 and Ty-1 and the chicken virus strain Ch-1 were

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avirulent for suckling mice (Mori et al., 2001, 2002). Although PO-13 was detectable in the intestines of suckling mice until 48 h postinoculation, Ty-3 was almost undetectable in the intestines from 4 h postinoculation (Mori et al., 2001). Because some of these viruses were still detected in the stomach at 4 h postinoculation (Mori et al., unpublished data), all of Ty-3 must be not excreted at that time. It is assumed that Ty-3 is immediately inactivated by some host factor(s) in gastrointestinal tracts of suckling mice and is avirulent for suckling mice as a consequence of absent or little exposure of Ty-3 to enterocytes. Thus, this phenomenon seems to be one of the mechanisms underlying virulence and host range restriction of avian rotaviruses.

Rotavirus particles have a triple-layered protein capsid that surrounds the genome of 11 segments of double-stranded RNA. Structural proteins, VP4 and VP7, have been shown to be determinants of host range (Ciarlet et al., 1998; Hoshino et al., 1995). VP4 is an unglycosylated protein and forms dimeric spikes that project out from the outer layers of mature virions, which are mainly composed of the glycoprotein VP7 (Both et al., 1994). Moreover, VP4 has been shown to be a determinant of several important functions, such as cell attachment, entry into cells, hemagglutination, and neutralization (Dunn et al., 1995; Estes and Cohen, 1989; Kaljot et al., 1988; Ludert et al., 1996). It is conceivable that VP7 is also involved in virus attachment to cells or entry into cells because this protein can induce a neutralizing antibody response against rotaviruses in animals (Fiore et al., 1995). Although both VP4 and VP7 exist in the outermost part of rotavirus particles, it is thought that they are first affected by a milieu of gastrointestinal tracts. From these points, it is suggests that VP4 and VP7 may be determinants of virulence and host range restriction of avian rotaviruses for mice.

In this study, to identify the determinants of the immediate inactivation of the avirulent strain Ty-3, we prepared PO-13 × Ty-3 reassortant strains with VP4 and/or VP7 genes, which were derived from the opposite parent virus strains. Experiments in which suckling mice were inoculated with the reassortant strains demonstrated that the VP7 gene derived from PO-13, regardless of derivation of the VP4 gene, was related to the immediate inactivation of Ty-3 in the gastrointestinal tract and that the VP4 gene also contributes to the virulence to suckling mice.

## Results

### *The inactivating effect against the avirulent avian strains for suckling mice in their stomachs and intestines*

At the first step, a virulent, PO-13, and three avirulent avian strains, Ty-3, Ty-1, and Ch-1, for suckling mice were orally inoculated to 3-day-old mice, and the virus titers in intestines were measured at 4 h postinoculation. Figure 1A indicated the recovered rate of each avian strain. Although

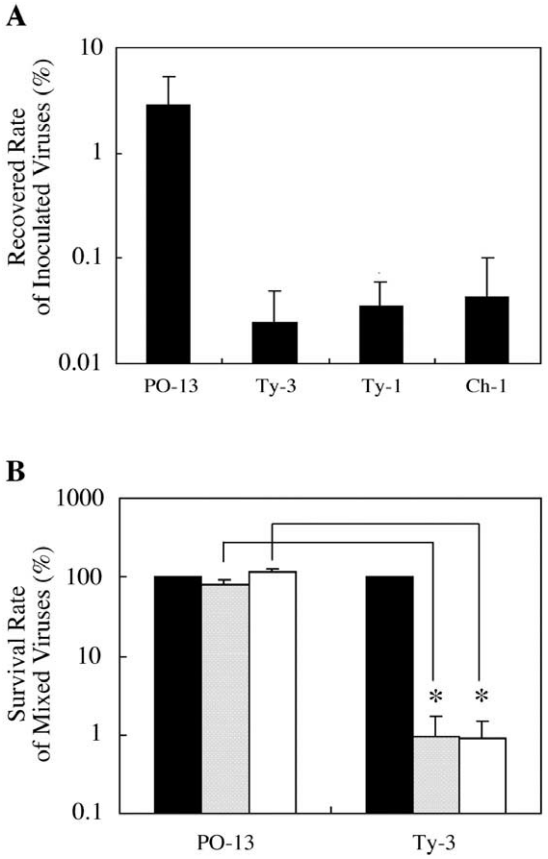


Fig. 1. Avian rotaviruses avilurent for suckling mice were immediately inactivated in gastrointestinal tracts of suckling mice. (A) Recovered rate of avian rotaviruses inoculated orally to suckling mice. Approximately $10^6$ FFU of avian rotaviruses were inoculated to 3-day-old mice ($n = 3$), and virus titers in intestines at 4 h postinoculation were measured. Recovered rate of each virus was expressed as FFU per intestine per that of the inoculated virus. Titers less than limit of virus detection, $10^{2.4}$ FFU, were calculated as $10^{2.0}$ FFU. (B) Survival rate of avian rotaviruses mixed with homogenate of stomachs or intestines extirpated from suckling mice. PO-13 or Ty-3 ($10^7$ FFU/ml) was mixed with equal weight of homogenates of stomachs (shaded bars) or intestines (white bars) extirpated from 3- or 4-day-old suckling mice and then incubated at 37°C for 4 h. As a control, PO-13 or Ty-3 was mixed with the same volume of Eagle's minimal essential medium (black bars) and incubated. Virus titers were determined by the fluorescent focus assay, and survival rates of viruses were expressed as FFU per gram of mixture with homogenates divided by FFU per milliliter of control. The arithmetic mean ± standard deviation of three experiments is shown. Asterisks indicate significant differences ($P < 0.01$).

3% of the inoculated PO-13 was recovered from intestines, only 0.025–0.043% of the avirulent strains were recovered. This result suggests that the avirulent strains are immediately inactivated in a milieu of gastrointestinal tracts of suckling mice. However, there were a possibility that the infectivity of PO-13, but not those of the other strains, was increased in a milieu of gastrointestinal tracts. Therefore, to further confirm this phenomenon, PO-13 and Ty-3, respectively, were mixed in vitro with equal volumes of either homogenates of stomachs or intestines extirpated from suckling mice and incubated at 37°C for 4 h, and the survival rate of each virus was determined (Fig. 1B). The

Table 1
Gene segment assignments and vilurences in suckling mice of a series of PO-13 × Ty-3 reassortants

| Strain | Gene segment assignments[a] | | | | | | | | | | | $DD_{50}$[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 (VP1) | 2 (VP2) | 3 (VP3) | 4 (VP4) | 5 (NSP1) | 6 (VP6) | 7 (NSP3) | 8 (NSP2) | 9 (VP7) | 10 (NSP4) | 11 (NSP5) | |
| PO-13 | P | P | P | P | P | P | P | P | P | P | P | $8.1 \times 10^3$ |
| Ty-3 | T | T | T | T | T | T | T | T | T | T | T | $>5.6 \times 10^7$ |
| P-T4P7 | P | P | P | T | P | P | P | P | P | P | P | $8.4 \times 10^7$ |
| P-P4T7 | P | P | P | P | P | P | P | P | T | P | P | $4.2 \times 10^7$ |
| P-T4T7 | P | P | P | T | P | P | P | P | T | P | P | $8.9 \times 10^7$ |
| T-P4T7 | T | T | T | P | T | T | T | T | T | T | T | $5.2 \times 10^7$ |
| T-T4P7 | T | T | T | T | T | T | T | T | P | T | T | $5.6 \times 10^7$ |
| T-P4P7 | T | T | T | P | T | T | T | T | P | T | T | $3.7 \times 10^5$ |

[a] Reassortant gene segment assignments were determined by comparisons of migration patterns of gene segments in SDS–PAGE with those of the parent strains PO-13 and Ty-3 and by comparisons of patterns of RT-PCR-RFLPs that were able to distinguish PO-13 and Ty-3 genes of VP1 (segment 1), VP6 (segment 6), NSP3 (segment 7), NSP2 (segment 8 of PO-13), VP7 (segment 9 of PO-13), NSP4 (segment 10), and NSP5 (segment 11). Because it was not possible to determine whether gene segments 8 and 9 of Ty-3 coded either NSP2 or VP7, gene segment 9 of Ty-3 is shown for the sake of convenience as Ty-3 VP7 gene. The genes derived from PO-13 and Ty-3 are indicated as P and T, respectively.

[b] $DD_{50}$ was measured in 3- or 4-day-old mice.

infective titers of PO-13 were not affected by incubation with them. In contrast, the infective titers of Ty-3 approximately 100-fold decreased by incubation with them, with significant differences by Student's *t* test ($P < 0.01$) from those of PO-13. These results suggested that the infectivity of PO-13 was not affected but Ty-3 was exactly inactivated in both stomachs and intestines of suckling mice.

### *Production of PO-13 × Ty-3 reassortant strains that have VP4 and/or VP7 genes derived from the opposite virus strains*

The viral outer capsid proteins, VP4 and/or VP7, could be related to the inactivation of Ty-3 in a milieu of gastrointestinal tracts of suckling mice because both proteins exist on the surfaces of viral particles (Estes and Cohen, 1989). To determine the viral protein(s) responsible to it, six PO-13 × Ty-3 reassortant strains that have only VP4 or VP7 or both VP4 and VP7 genes derived from the opposite parent virus strains were isolated from MA104 cells co-infected with PO-13 and Ty-3 strains (Table 1). Migration patterns of each gene segment of these strains in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed clearly the origins of the gene segments 2, 3, 4, and 5, encoding VP2, VP3, VP4, and NSP1, respectively (Fig. 2 and Table 1).

The origins of the remaining genes 1, 6, 7, 8, 9, 10, and 11, undistinguished by SDS–PAGE, were determined by reverse transcription, polymerase chain reaction, and restriction fragment length polymorphisms (RT-PCR-RFLP) (Fig. 3 and data not shown). The cDNAs of the VP7 genes (genes segment 8 or 9) derived from both PO-13 and Ty-3 were amplified to 930 and 931 bp in length, respectively (Fig. 3). According to sequences (accession numbers, PO-13: D82979 and Ty-3: AB080737), it seems that RT–PCR products of the VP7 genes of PO-13 and Ty-3 are digested by the restriction enzyme *Dra*I into three (604, 294, and 32 bp) and two segments (898 and 33 bp) respectively. Although the smallest segments were unable to be visualized, the VP7 genes of PO-13 and Ty-3 clearly were distinguished in 2% agarose gel (Fig. 3). Thus, the RFLP patterns of the reassortant strains revealed the origins of these VP7 genes (Fig. 3 and Table 1). As well as the VP7 gene, the

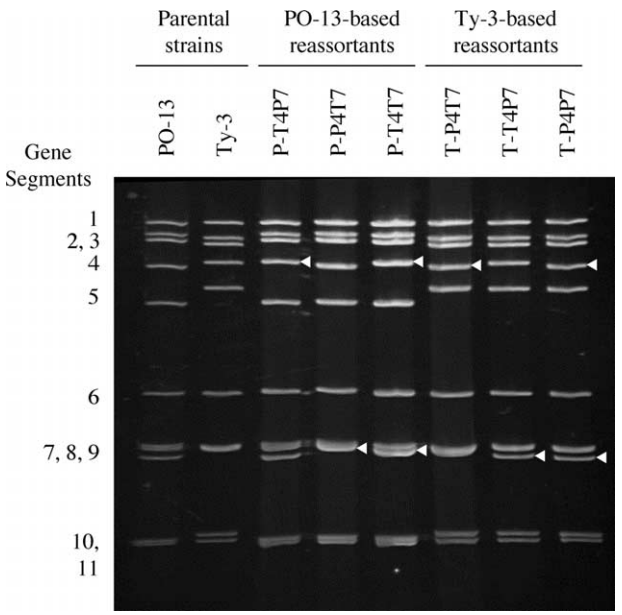


Fig. 2. Electrophoretic migration patterns of genomic RNA segments from the parental strains PO-13 and Ty-3 and six PO-13 × Ty-3 reassortant strains on 10% polyacrylamide gel. Gene segments are numbered on the left. Arrowheads show reassorted segments. P-T4P7 and T-P4T7 were reassorted on segment 4 (VP4 gene), and P-P4T7 and T-T4P7 were done on segment 9 (VP7 gene). P-T4T7 and T-P4P7 were replaced on both segments 4 and 9.

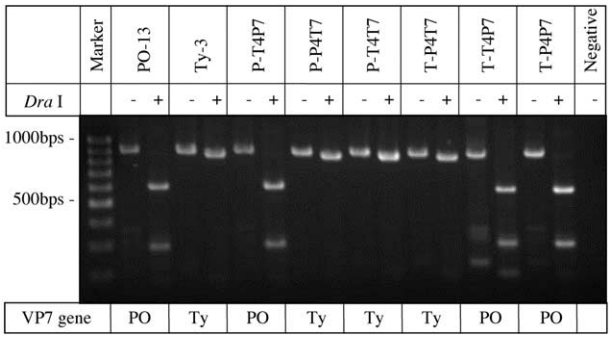


Fig. 3. RT-PCR-RFLP patterns of VP7 genes. The RT–PCR products of VP7 genes derived from the parental strains, PO-13 and Ty-3, and the reassortant strains were electrophoresed in 2% agarose gel with (+) or without (−) digestion by a restriction enzyme, *Dra*I. The RT–PCR products of the VP7 genes derived from PO-13 (930 bp) and Ty-3 (931 bp) were digested into three segments (604, 294, and 32 bp) and two segments (898 and 33 bp), respectively. Negative indicates a negative control in RT–PCR, PO and Ty indicate PO-13 and Ty-3, respectively.

other genes of these strains were also determined in RT-PCR-RFLPs (data not shown).

In addition, IF assays using anti-PO-13 antibodies were performed to confirm the phenotypes of the reassortant strains (Table 2). The anti-PO-13 rabbit serum, which reacted with both cells infected with PO-13 and cells infected with Ty-3, was found to react with cells infected with all reassortant strains. On the other hand, the anti-VP4 and VP7 monoclonal antibodies (MAb), P3-31 and P3-5, which reacted with only cells infected with PO-13, were found to react with cells infected with the reassortant strains that had the VP4 and/or VP7 genes derived from PO-13, respectively. Therefore, these results indicated that the six reassortant strains had intended VP4 and VP7 phenotypes.

### *Pathogenicities of PO-13 × Ty-3 reassortant strains for suckling mice*

To compare the virulences of the PO-13 × Ty-3 reassortant strains for suckling mice, the 50% diarrhea-inducing doses ($DD_{50}$) of these viruses were determined in 3- or 4-day-old ddY mice (Table 1). The $DD_{50}$ value of each reassortant strain with the genetic background of PO-13 (P-T4P7, P-P4T7, and P-T4T7) was approximately $10^4$-fold higher than that of PO-13. The monoreassortant strains based on Ty-3, T-P4T7 and T-T4P7, showed little change in virulence of Ty-3, with $DD_{50}$ values of $5.2 \times 10^7$ focus-forming units (FFU) and $5.6 \times 10^7$ FFU, respectively. Only T-P4P7 was found to have an intermediate virulence, with $DD_{50}$ of $3.7 \times 10^5$ FFU. Inoculation of $10^6$ FFU of T-P4P7 induced diarrhea in 12 of 15 mice, whereas none of 15 mice inoculated $10^6$ FFU of Ty-3 suffered from disease, indicating the significant difference by chi-square test ($\chi^2 = 16.8$, $p < 0.01$). These results showed that the difference between the pathogenicities of PO-13 and Ty-3 for suckling mice is mainly regulated by both VP4 and VP7.

### *Kinetics of PO-13 × Ty-3 reassortant strains in intestines of suckling mice*

To determine which protein, VP4 or VP7, is related to Ty-3 inactivation in a milieu of gastrointestinal tracts of suckling mice, each reassortant strain was orally inoculated to suckling mice with approximately $10^6$ FFU, and virus titers in the intestines were measured (Fig. 4). Inoculation of $10^6$ FFU of virulent strains, PO-13 and T-P4P7, but not avirulent strains, induced diarrhea for suckling mice (Table 1). PO-13 showed a constant titer of about $10^4$ FFU at 4 to 24 h postinoculation. On the other hand, as described previously (Mori et al., 2001), Ty-3 was not detected in intestines from an early stage until 4 h postinoculation (Fig. 4A). Among the six reassortant strains, all strains possessing the Ty-3 VP7 gene (P-P4T7, P-T4T7, and T-P4T7), regardless of the origin of the VP4 gene, were undetectable until 4 h postinoculation as was Ty-3 (Fig. 4B and C). On the other hand, all strains (P-T4P7, T-T4P7 and T-P4P7) possessing the PO-13 VP7 gene were detected at 4 h postinoculation. These results indicate that Ty-3 VP7 is mainly related to the virus inactivation in gastrointestinal tracts of suckling mice. Among the reassortant strains with PO-13 VP7, the titers of P-T4P7 and T-T4P7 were under the detection limit at 24 h postinoculation (Fig. 4B and C). However, T-P4P7, which had a high degree of virulence for suckling mice, was able to be detected until 24 h postinoculation (Fig. 4C). It is not clear whether T-P4P7 is able to replicate in enterocytes of suckling mice, because the titer of T-P4P7 continuously decreased throughout the period of the examination. It has been reported that replication of PO-13 in suckling mice peaked at 24 h postinoculation (Mori et al., 2001). Because the mean titer of T-P4P7 at 24 h postinoculation was only about sixfold lower than that of PO-13, with not a significant difference by Student's *t* test ($P = 0.095$), T-P4P7 might infect and replicate in enterocytes of suckling mice, resulting in diarrhea.

Table 2
Reactivities of PO-13, Ty-3, and six reassortant strains with anti-PO-13 antibodies in IF assays

| Strain | IF titers[a] | | |
|---|---|---|---|
| | Anti-PO-13 rabbit serum | Anti-VP4 MAb P3-31 | Anti-VP7 MAb P3-5 |
| PO-13 | 8,000 | 8,000 | 32,000 |
| Ty-3 | 8,000 | <100 | <100 |
| P-T4P7 | 8,000 | <100 | 32,000 |
| P-P4T7 | 8,000 | 2,000 | <100 |
| P-T4T7 | 8,000 | <100 | <100 |
| T-P4T7 | 4,000 | 4,000 | <100 |
| T-T4P7 | 8,000 | <100 | 16,000 |
| T-P4P7 | 16,000 | 2,000 | 16,000 |

[a] IF titers were expressed as the reciprocal of the highest antibodies dilution that reacted with MA104 cells infected with the indicated viruses.

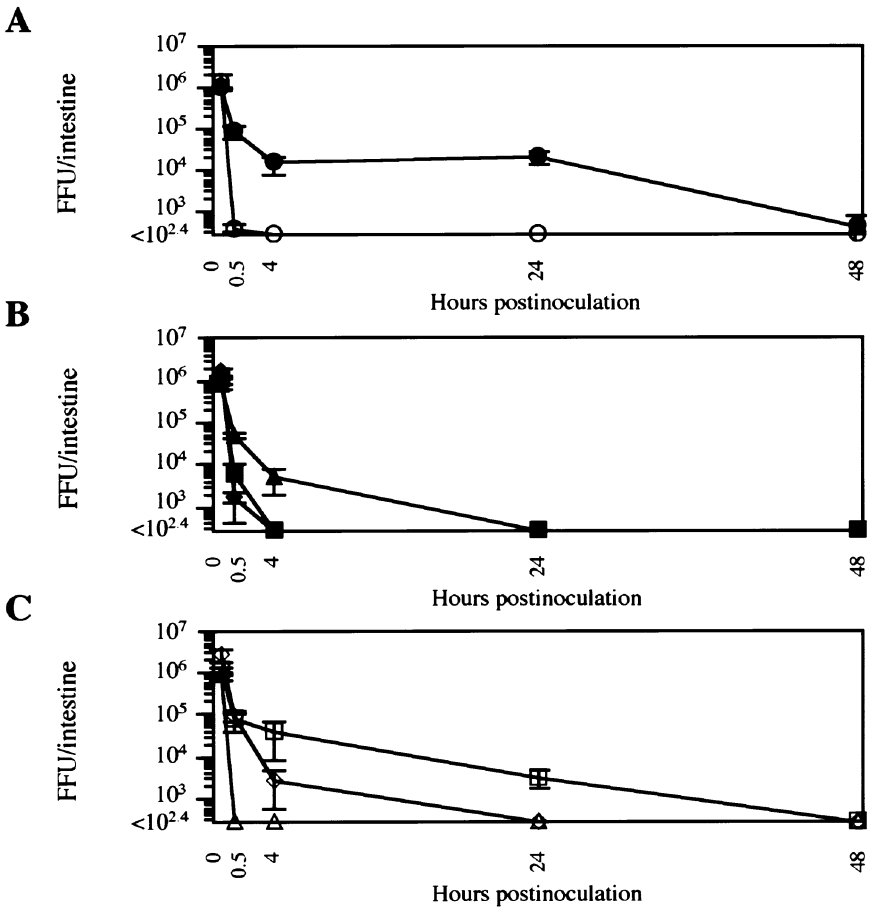


Fig. 4. The kinetics of the parental avian rotaviruses PO-13 (●) and Ty-3 (○) (A); the PO-13-based reassortant strains P-T4P7 (▲), P-P4T7 (◆), and P-T4T7 (■) (B): and the Ty-3-based reassortant strains T-P4T7 (△), T-T4P7 (◇), and T-P4P7 (□) (C) in intestines of suckling mice inoculated at 3 or 4 days of age. Suckling ddY mice were orally inoculated with approximately $10^6$ FFU of each rotavirus. The inoculated mice were sacrificed under diethyl ether anesthesia, and the entire intestines were collected at the indicated hours postinoculation. Titers of infectious virus in the intestines were determined by a focus-forming assay. The limit of virus detection was $10^{2.4}$ FFU. The arithmetic mean ± standard deviation of three mice is shown. Titers less than $10^{2.4}$ FFU were calculated as $10^{2.0}$ FFU.

## Discussion

Many studies have shown that there are host range restrictions of rotaviruses to heterologous animals, that is, almost all heterologous strains cannot replicate or replicate more limitedly than homologous strains (Bell et al., 1987; Ciarlet et al., 1998, 2000; Mori et al., 2001; Ramig, 1988), but the mechanisms underlying these restrictions are not clearly understood. Recently, we found that the pigeon virus strain PO-13, which is virulent for suckling mice, existed for a long time in gastrointestinal tracts of them, whereas the turkey virus strain Ty-3, which is avirulent for them, immediately lost its infectivity in these gastrointestinal tracts (Mori et al., 2001). The other avirulent strains, Ty-1 and Ch-1, for suckling mice were also inactivated in them (Fig. 1A). Furthermore, we indicated that incubation with either homogenates of stomachs or intestines extirpated from suckling mice greatly reduced the infectivity of Ty-3, but not that of PO-13 (Fig. 1B). It is maybe suggested that the avirulences of Ty-3, Ty-1, and Ch-1 for heterologous suckling mice were caused by the inactivation of viruses in these gastrointestinal tracts. In this context, these inactivations could be one of the determinants of the host range restriction. Bell et al. (1987) and Ramig (1988) reported that some heterologous mammalian rotaviruses immediately lost their infectivities and did not induce diarrhea after oral inoculation to suckling mice. Although they explained that these phenomena were caused by little or no replication of the viruses in suckling mice, these viruses might be also inactivated in the gastrointestinal tract.

Because the mucosal surface of the gastrointestinal tract is a major interface between an animal and its environment, there are many specific and nonspecific defense systems against pathogens, such as parasites, bacteria, and viruses, in the gastrointestinal tract. It seems that these specific defenses were not involved in degrees of the inactivation of avian rotaviruses in mice because all of the mice used in the experiments had been naive for rotavirus infection. On the other hand, it is known that there are various defense mechanisms nonspecific for pathogens, such as proteolysis by

low pH or enzymes in the stomach; a physical barrier by mucus; excretion of pathogens by cellular turnover and peristalsis of the intestine; maintenance of homeostasis by intestinal microflora; and existence of antimicrobial substances such as bile salts, lactoferrin, lysozyme, and lactoperoxidase (Stokes and Bourne, 1989). Previous studies have shown that several substances can inhibit rotaviruses infection (Bass et al., 1992; Beisner et al., 1998; Coulson et al., 1997; Guerrero et al., 2000; Reading et al., 1998; Sato et al., 1995; Superti et al., 2001; Takahashi et al., 2001; Yolken et al., 1992, 1994). These inhibiting activities are explained as binding to outer capsid proteins of rotaviruses, resulting in competition of interactions between viruses and their target cells (Yolken et al., 1992, 1994 and Superti et al., 2001) or as enzymatic destruction of viral structural proteins (Bass et al., 1992; Sato et al., 1995).

Although there are a few reports of VP7 being related to host range restriction (Ciarlet et al., 1998; Hoshino et al., 1995), it is not clear how VP7 contributes this restriction. The present experiments using PO-13 × Ty-3 reassortant strains demonstrated that VP7 is mainly involved in survival and inactivation of avian rotaviruses in gastrointestinal tracts of suckling mice (Fig. 4). Several studies have shown that VP4–VP7 interactions in reassortants affect the expression of phenotypes of VP4, such as receptor binding (Mendez et al., 1996), and binding with neutralizing antibody or conformational structures (Pesavento et al., 2003). However, these influences might not be very important to the inactivation of Ty-3 because anti-PO-13 VP4 MAb P3-31 was able to react with PO-13 VP4, regardless of the origins of VP7 (Table 2). It has been reported that most inactivators can react with VP4 except for bovine collectins (Reading et al., 1998), murine $\alpha$1-antitrypsin (Beisner et al., 1998), and extract from *Stevia rebaudiana* (Takahashi et al., 2001), which react with VP7. It is not known at present whether there are similar materials to these VP7-binding substances in gastrointestinal tracts of suckling mice. In this study, the mechanisms underlying the Ty-3 inactivation, dependent on its VP7, in gastrointestinal tracts of suckling mice remains still unclear. Thus, further studies are needed to resolve this question.

In this study, we indicated that the monoreassortment by the virulence-associate gene, VP4 or VP7, caused attenuation of the virulent strain, PO-13, and the direassortment of VP4 and VP7 genes gave a virulence to the avirulent strain, Ty-3. This result is similar to the report by Hoshino et al., (1995) that identified four genes associate to virulence and host range restriction in piglets using porcine and human reassortant strains. Taken together, it is suggested that each virulence-associated gene plays a distinct role(s) in a series of continuous stage of replication, including access, attachment, and penetration to target cells; uncoating; transcription; translation; assembly; budding of newly assembled virus particles; and maybe enterotoxin release (Zhang et al., 2000).

Another virulence-associate protein, VP4, is shown to be a determinant of several important functions, such as cell attachment and entry into cells (Kaljot et al., 1988; Ludert et al., 1996). The preliminary study suggested that PO-13 and Ty-3 recognize different receptors, because treatment of MA104 cells with purified VP8 of PO-13, which is one of the proteins generated by cleavage of VP4, inhibited the infection of PO-13 but not that of Ty-3 (M. Sugiyama et al., unpublished data). The degree of expression of each receptor for PO-13 and Ty-3 in suckling mice might regulate their infectivities.

The $DD_{50}$ value of T-P4P7, which had the same set of outer capsid proteins as that of PO-13, was 46-fold higher than that of PO-13, suggesting that this difference is related to congenialities between proteins derived from PO-13 and Ty-3 or other pathogenic viral proteins, such as VP3, NSP1, and NSP4 (Ciarlet et al., 2000; Dunn et al., 1994; Horie et al., 1999; Hoshino et al., 1995; Ito et al., 2001). In this study, other determinants in addition to VP4 and VP7 in pathogenicity were not identified because only reassortant strains of VP4 and VP7 genes were produced to decide the determinant of the virus inactivation. However, the difference between virulences of PO-13 and T-P4P7 might be reflected to these NSP4s. Although all of the NSP4s derived from the avian rotaviruses, PO-13, Ty-3, Ty-1, and Ch-1, had enterotoxigenic activity in suckling mice, the $DD_{50}$ value of Ty-3 NSP4 was slightly higher than that of PO-13 NSP4 (Mori et al., 2002).

PO-13 is the only avian rotavirus of which the virulence for suckling mice has been confirmed. The deduced amino acid sequences of PO-13 VP7 share only 70, 83, and 74% homologies with those of the avirulent strains Ty-3, Ty-1, and Ch-1 (determined in this study; accession number AB080738), respectively, suggesting that PO-13 belongs to different G serotypes from the other strains (Rohwedder et al., 1997). Because homology in VP7 between PO-13 and the avianlike bovine rotavirus 993/83 is 99% (Rohwedder et al., 1997), PO-13 and 993/83 could be considered to share the same G serotype and similar functions for virulence.

This study has demonstrated that the difference between degrees of virulence of PO-13 and Ty-3 for suckling mice is dependent on their outer capsid proteins, VP7 and VP4. Although further studies on substances that react with VP7 are needed to elucidate the mechanisms underlying the immediate disappearance of rotaviruses in gastrointestinal tracts of suckling mice, virus inactivation through VP7 in the gastrointestinal tract is thought to be one of the mechanisms underlying the host range restriction of rotaviruses.

## Materials and methods

### *Viruses*

The pigeon rotavirus strain, PO-13, was isolated from Japanese pigeon feces using rhesus monkey kidney MA104

cells (Minamoto et al., 1988) and was passaged 15 times in MA104 cells and twice in bovine kidney MDBK cells, containing the plaque cloning. The turkey rotavirus strains, Ty-3 and Ty-1, and the chicken rotavirus strain, Ch-1, were isolated using chicken embryo fibroblast cells and chick kidney cells in the UK (McNulty et al., 1980). These turkey and chicken viruses were provided by Dr. McNulty (Veterinary Research Laboratories, UK) and were passaged three to five times in MA104 cells in our laboratory, containing the plaque cloning. All of PO-13 × Ty-3 reassortant strains were grown in MA104 cells with typsin. To prepare stock viruses of Ty-3 and PO-13 × Ty-3 reassortant strains with high titers, a culture fluid of MA104 cells infected with each virus was concentrated by ultracentrifugation as described previously (Mori et al., 2001). Virus titers were determined by fluorescent focus assays in MA104 cells and expressed as FFU per milliliter as described previously (Mori et al., 2001).

*Animals*

The ddY strain of closed colony mice was used in this study. As well as inbred Balb/c strain, this strain was susceptible to PO-13 infection but not to Ty-3, Ty-1, and Ch-1 infections (Mori et al., 2001, 2002). Pregnant ddY mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed individually in cages. After delivery, blood samples were taken from each dam, and serum antibody was checked by ELISA using PO-13 particles as antigens by the modified method of Burns et al. (1995). All of the dams checked were seronegative (titer: $<200$). The litter was kept with the dam throughout the course of the experiments.

*Animal inoculation*

Inoculation of viruses to suckling mice was performed as described previously (Mori et al., 2001). Briefly, each mouse in litters of 3- to 4-day-old suckling mice was given 20 $\mu$l of virus by oral gavage. Diarrhea was diagnosed by gentle abdominal palpation at various times after inoculation. The state of the stool was classified into three categories: watery diarrhea, diarrhea with solid matters, loose yellow stool, and ordinary stool. Watery diarrhea and diarrhea with solid matters were considered as diarrhea. To determine $DD_{50}$, more than eight mice, which are from distinct two litters at least, were inoculated with each dilution of viruses. Based on the results, the $DD_{50}$ values were calculated by the method of Reed and Müench (1938).

*Determination of virus titers in the intestines of suckling mice*

The kinetics of each inoculated virus in the intestines of suckling mice was determined as described previously (Mori et al., 2001). At various times after inoculation, three mice inoculated with each virus were sacrificed under diethyl ether anesthesia, and intestine samples were collected. The intestines were weighed and stored at $-80$°C until use. The intestines were homogenated, treated with AK-225 (Asahi Glass, Japan) as an alternate of fluorocarbon, and centrifuged for 10 min at 750*g* at 4°C. After treatment with typsin, virus titers in the resulting aqueous layers were determined by fluorescent focus assays and expressed as FFU per intestine. Recovered rate from intestine of each virus was expressed as FFU per intestine divided by that of the inoculated virus.

*Virus-inactivation assays*

The inactivating effects of homogenates of stomachs and intestines of suckling mice against avian rotaviruses were determined as follows. In brief, $10^7$ FFU/ml of PO-13 or Ty-3 was mixed with equal weight of homogenates of stomachs or intestines extirpated from 3- or 4-day-old suckling mice. As a control, PO-13 and Ty-3 were mixed with the same volume of Eagle's minimal essential medium. After incubating at 37°C for 4 h, these mixtures were treated with AK-225 and centrifuged for 10 min at 750*g* at 4°C. The resulting aqueous layers were collected as an inoculum for MA104 cells. Virus titers were determined by the fluorescent focus assay, and ratios of survival virus titer were expressed as FFU per gram of mixture divided by FFU/ml of control.

*Production of PO-13 × Ty-3 reassortant strains*

Reassortant viruses were prepared by the method of Offit et al. (1986). Briefly, MA104 cells were co-infected with PO-13 (multiplicities of infection of 1, 2, 4, or 8) and Ty-3 (multiplicities of infection of 1). After 1- or 2-day incubation at 37°C, the culture fluid of the co-infected MA104 cells was harvested. Progeny viruses were plaque-purified once in MA104 cells. The required reassortant strains were selected by SDS–PAGE and RT-PCR-RFLP of the genomic RNA and IF assays in infected cells and then were further plaque-purified twice in MA104 cells. Reassortant strains are designated as combinations of the name of the parent virus with a majority of RNA segments and the names of origins of the VP4 and VP7 genes. For example, the reassortant strain P-T4T7 has the VP4 and VP7 genes derived from Ty-3 with the genetic background of PO-13 (P, PO-13; T, Ty-3; 4, VP4; 7, VP7).

*SDS–PAGE of genomic RNA from rotaviruses*

Reassortant viruses were selected by comparisons of the migration patterns of genome segments with those of the parent strains PO-13 and Ty-3 by SDS–PAGE. Briefly, genomic RNAs were extracted from the concentrated viruses using ISOGEN (Nippon gene, Japan) and were separated in a 10% polyacrylamide gel by the method of

Laemmli (1970). The gel was stained with ethidium bromide and observed under a UV light transilluminator.

*RT-PCR-RFLP*

Gene segments 1 (VP1 gene), 6 (VP6 gene), 7 (NSP3 gene), 8 (NSP2 or VP7 gene), 9 (VP7 or NSP2 gene), 10 (NSP4 gene), and 11 (NSP5 gene), which were not able to be distinguished by SDS–PAGE, were determined by RT-PCR-RFLP. Briefly, the cDNAs corresponding to each gene segment from parent viruses and reassortant viruses were generated by Ready-To-Go You-Prime First Strand Beads (Amersham, USA) with the primer mixture with VP1-1: GGCTATTAAAGCTATACGATGGGG (VP1 gene); VP6-1; GGTCACATCCTCTCACT (VP6 gene); NSP3-1: TTTAACATCAAGTTAGCTTTTAACT (NSP3 gene); NSP2-1: GGCTTTTAAAGCGTCTCGGTCG (NSP2 gene); VP7-1: CCGGATCCGGTCACATC (VP7 gene); NSP4-1: TTTAACATCAAGTTAGCTTTTAACT (NSP4 gene); and NSP5-1: GGTCACAAAACGGGAGTGG (NSP5 gene). The each generated cDNA was amplified by PCR with the sense and antisense primer pair, VP1-1 and VP1-2: GTTCAAGTTCTTCCGTTGTG (VP1 gene); VP6-2: TTTCAGACTGGAGGAATTGG and VP6-3: GGAAATACCGGTCCAACTGG (VP6 gene); NSP3-2: ATTTGATTTTGGATGATTCTGGTGT and NSP3-1 (NSP3 gene); NSP2-1 and NSP2-2: GGTCACATAAAGCGCTTTCAATTC (NSP2 gene); VP7-2: CAGTAATTTCGTTTCTCACCG and VP7-3: ACTTGCCACCATTTCTTCCAAT (VP7 gene); NSP4-2: ATTTGATTTTGGATGATTCTGGTGT and NSP4-1 (NSP4 gene); or NSP5-2: GGCTTTTAAAGCGCTACAGTG and NSP5-1 (NSP5 gene). The PCR products were digested by the restriction enzymes *Ase*I for VP1 gene; *Dra*I for VP7 gene; *Hinc*II for NSP4 gene; and *Dde*I for VP6, NSP3, NSP2, and NSP5 genes. The RFLP patterns were determined by comparisons with those of the parent strains PO-13 and Ty-3 on electrophoresis.

*IF assays*

The VP4 and VP7 phenotypes of reassortant strains were determined by IF assays as described previously (Minamoto et al., 1993). Briefly, MA104 cells were inoculated with rotaviruses and incubated for 1 day. The infected cells were fixed with 4% paraformaldehyde and methanol and were then stained with the anti-PO-13 rabbit serum or anti-PO-13 VP4 MAb P3-31 (Rohwedder et al., 1997) or anti-PO-13 VP7 MAb P3-5 and subsequently with fluorescein-conjugated antirabbit immunoglobulin or goat antimouse immunoglobulin G (Cappel, USA). These MAbs failed to reacted with Ty-3-infected cells. IF titers were expressed as the reciprocal of the highest antibodies dilution that reacted with MA104 cells infected with each virus.

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